ISOLATION, CHARACTERIZATION AND EVALUATION OF GROWTH POTENTIAL OF PLANT GROWTH PROMOTING RHIZOBACTERIA FROM AGRICULTURAL FIELDS IN AGRA

Synopsis of the proposed work for

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Submitted by

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Introduction

Escalating population coupled with shrinkage of aerable land due to urbanization and industrialization have posed a serious problem to food security. Consequently, there is an immense pressure on agricultural lands for higher crop production. To meet the challenge, enormous amounts of chemical fertilizers are utilized, which are not only costly but also create environmental problems such as, deterioration of soil quality, reduced growth of microorganism and eutrophication of aquatic ecosystems (Vessey, 2003; Adesemoye *et al.,* 2009). Further, there are waste stretches of non aerable land affected either by salinity or alkinity (Ritzewma *et al.,* 2008)

Alternately, soil fertility has been maintained by traditional practices as crop rotation or through organic farming practices. Besides, legumes crop cultivation is a common practice to increase soil nitrogen. Currently stress for the use of biofertilizers to improve soil fertility and promoting plant growth is on the increase (Adesemoye *et al.,* 2009).

Most soils have an array of microorganisms and a rough estimate suggests that one gram of soil contains approximately 90 million bacteria, 4 million actinomycetes, 2 lakh fungi, 30 thousand algae, 500 protozoa and 30 nematodes (Alexander, 1991). The rhizosphere is represented by a zone of soil that surrounds the plant roots and supports a large active group of microorganisms, of which only 1-2% promote plant growth (Antoun and Kloepper, 2001). Plant growth promoting rhizobacteria (PGPR), also known as beneficial soil bacteria of rhizosphere, enhance growth of host plant through direct or indirect mechanisms (Kloepper and Scroth, 1978). The interactions between PGPR and roots in the rhizosphere can influence soil fertility (Dastager *et al*., 2011). The PGPR include a wide range of root colonizing bacteria having the ability to enhance plant growth and reduce or suppress growth of deleterious plant pathogenic fungi, bacteria and nematodes by inducing systemic resistance (Van Loon *et al.,* 1998; Kloepper *et al*., 2004).

PGPR also promote growth in plants by producing phytohormones (Sivasakthi *et al*., 2014). Some rhizobacteria produce phytohormones as auxin, gibberellins and cytokinins (Rupaedah *et al,* 2014). Indole-3-acetic acid (IAA) plays an important role in root initiation, cell division and cell

enlargement (Salisbury, 1994). Experiments have demonstrated that in rhizobacteria elevated level of the precursor L-tryptophan could increase auxin production (Ahmad *et al.,* 2008). Auxin production by rhizobacteria lead to changes in root morphology by proliferation and elongation of adventitious and lateral roots of the plant. Rhizobacteria belonging to the genera *Azospirillum, Pseudomonas, Xanthomonas, Rhizobium, Alcaligenes faecalis*, *Enterobacter cloacae, Azetobacter diazotrophicus* and *Bradyrhizobium japonicum* have been shown to produce auxins which stimulate plant growth (Glick and Patten, 1996). Cytokinins are known to promote cell division, cell enlargement and tissue expansion in certain plant parts (Salisbury, 1994). Some rhizobacteria that produce cytokinin are *Bacillus licheniformis, Bacillus subtilis, Pseudomonas aeruginosa, Bacillus megaterium, Bacillus cereus* and *Bacillus subtilis* (Hussain and Hasnain, 2009). Certain PGPR strains are now known to promote plant growth by decreasing ethylene level in plants. These bacteria such as *Pseudomonas* sp. are capable of bringing about this growth promotion by producing 1-aminocyclopropane-1-carboxylate (ACC) deaminase that breaks down ACC into ammonia and α-Ketobutyrate and thus inhibiting ethylene production in stressed plants (Glick *et al*., 1998: Akhgar *et al*., 2014; Glick, 2014).

PGPR, such as *Pseudomonas* and *Bacillus,* are known to increase available soil phosphorus by converting insoluble phosphates into soluble forms (Puente *et al.,* 2004; Ponmurugan and Gopi, 2006; Sharma *et al*., 2013). The nitrogen fixing bacteria as *Rhizobium, Frankia, Azospirillum, Azotobactor, Azoarcus* and *Acetobacter* improve soil nitrogen content by fixing atmospheric nitrogen (Sivasakthi *et al.,* 2014).

Often iron, though present in soils in abundance, is not available to the plants as most of it is in the ferric form, Certain bacteria produce siderophore that have high affinity for ferric ions and chelate it. The chelated ferric ion get reduced to ferrous ion, which is readily taken up by plants (Vessey, 2003).

The ability of PGPR, to promote plant growth varie with the strain. There is a continuous search for more PGPR strains. This requires collection, identification and application to assess the PGPR strains. Identification of PGPR is based on morphological, biochemical and molecular mechanism. Molecular method involves amplification of the 16S regions of the conserved bacterial

DNA, and is specific to each bacteria, thus making each amplicon a unique sequence. These amplicons are sequenced and subjected to bioinformatics studies to develop a phylogenic tree and identification of the bacterial strain (Janda and Abbott, 2007).

In the U.S.A. several bioformulations of PGPR as powder soluble formula have been registerd. Some example of *Bacillus* based formulations are: BioYield, Companion, Ecoguard etc and those for *Pseudomonas* based are: AtEze, Bio-save, BightBan etc. (Figueiredo *et al*., 2010). In india as well PGPR based bio-fertilizers are fast gaining acceptability and popularity (Raj *et al*., 2005).

Objectives of present work

Taking the aforesaid views in due consideration the present study has been undertaken with the following objectives:

1. Isolation and characterization of plant growth promoting rhizobacteria from rhizospheric soil.
2. Standardization of media composition and evaluation of growth kinetics parameters of certain rhizobacteria.
3. In vitro screening of bacterial isolates for their plant growth promoting activities.
4. Effect of potential plant growth promoting rhizobacteria in certain crop plants.

Review of literature

One of the major challenges for the twenty-first century will be environmental pollution and sustainable crop production. Plant growth and yield are affected by different parameters including soil type, plant, and climate properties. Altering soil properties including physical, chemical, and biological ones can influence plant growth and yield. There are various methods to maintain soil qualities, resulting in enhancement of soil productivity. Plants require nutrients for their optimal growth and higher production. Among the different methods of enhancing nutrient quantity and availability for plant utilization is the use of chemical fertilization which has tremendously increased and also enhanced the environmental pollution. (Vessey 2003).

To search out the plant growth promotion mechanisms by PGPR, literaure reports are increasing at a rapid rate in order to identify the best PGPR strain for commercial biofertilizer (Bhattacharyya and Jha, 2012). PGPR interact with the host plant and enhance the nutrient uptake of host plants through biological N_2 fixation, increasing the availability of nutrients in the rhizosphere, inducing increases in root surface area, enhancing other beneficial symbioses of the host, combination of modes of action.

PGPR can increase the root surface area and length and promote the plant growth and development by the production of different phytohormones like indole-3-acetic acid (IAA), gibberellic acid and cytokinins. A large root surface area helps the plant to access more nutrients from soil and thus contribute to plant growth promotion (Vessey, 2003).

Dias *et al.* (2013) obtained seventy eight isolates on the basis of IAA and phosphate solublization traits. These strains were characterized on basis of biochemical and 16S rRNA sequencing. These strains belong to genus pseudomonas and rhizobium. All strains have ability of IAA production but phosphate solublization ability was found in only fifty nine strains. Seven strains were characterized as *Pseudomonas putida* by intermediate solubilization and IAA production which promote sufficient growth of kale plant.

Kumar *et al.* (2012) screened thirty rhizobacteria and identified all isolates on the basis of morphological and biochemical characterization which belong to genus *Acinetobacter,*

Bacillus, Enterobacter, Micrococcus and *Pseudomonas*. Twelve strains were found positive for phosphate solublization in which FBJ6 (*Psudomonas* sp.) showed highest phosphate solublization zone (20 mm) but IAA production was shown by all strains and FBK3 (*Micrococcus* sp.) shown high production (213.15 µg/ml). Two isolates (*Pseudomonas* sp. and *Bacillus* sp.) were found positive for HCN production. Three isolates were positive for ammonia production and ACC activity was shown by nine isolates. All isolates were found positive for catalase activity.

Sharma and Rai (2015) isolated twenty one strains and characterized on the basis of phenotypic and biochemical tests. Three isolates from twenty one isolates were selected for IAA production that gave gram negative, catalase positive and starch hydrolysis positive tests. The IAA production was found maximum (50.25 µg/ml) by P19 while P3 showed minimum (15.25 µg/ml). All isolates were found positive for siderophore production while phosphate solublization was showed by only P19. Tomato seedling showed maximum shoot length (8.6 cm), root length (3.0 cm), shoot fresh weight (0.104 g), root fresh weight (0.005 g), fresh plant weight (0.109 g) dry plant weight (0.018 g) as compared to control when consortium of P3, P9 and P19 were provided.

Mahbouba *et al.* (2013) screened bacteria from soil of wheat crop. All isolates were characterized by morphological and biochemical tests and screened for plant growth promoting traits as production of IAA, production of ammonia, and phosphate solubiliztion. These isolates showed maximum growth at 6.8 pH and broad range of temperature (28 and 37 °C). Six isolates were identified by 16S rDNA sequencing, they showed maximum relativity with *Azospirillum brasilense*. Eight isolates were identified as nitrogen fixer by the presence of *nif* H genes in their genome.

Gusain *et al.* (2015) screened bacteria from the soil of agricultural field of Garwal in Himachal Predesh. These isolates were characterized by the production of IAA, phosphate solublization and siderophore. Two bacterial isolates which have efficient phosphate

solublization activity, were identified as *Pseudomonas koreensis* (YB1 strain), *Anthrobacter nitroguajacolicus* (YB3 strain) and three isolates, producing high amount of IAA, were identified as *Klebsiella oxytoca* (YB2 strain) and two strains of *Anthrobacter nitroguajacolicus* (YB4 and YB5 strain) using 16S rRNA sequence analysis. *Klebsiella oxytoca* showed maximum production of IAA (334.44 µg/l) when culture medium supplemented with L-Tryptophan. Phosphate solublization ability was tested at three incubation tepmperature (4, 10 and 28 ˚C) of selected five bacterial strains. *Pseudomonas koreensis*, *Anthrobacter nitroguajacolicus* (YB4 strain) and *Klebsiella oxytoca* solubilize phosphate efficiently at 28˚C while at 10˚C only two strains, *Pseudomonas koreensis* and *Anthrobacter nitroguajacolicus* (YB4 strain) solubilize phosphate efficiently as compare to other strains. These five strains were tested on two rice varieties Swarna and Swarna sub 1. These strains showed efficient increment and phosphate uptake in these two varieties.

Sadiq and Ali (2013) isolated ten bacterial strains from the rhizosphere of *Mimordica charantia, Zea mays, Oryza sativa* and *hordium vulgare.* The significant increase for shoot length and number of roots were seen in wheat by inoculating rhizobacterial isolates *E. cloacae* (22%) and *B. cereus* (46%) over control. Highest IAA production was observed in *B. megaterium* (140 µg/ml). It was observed that rhizobacteria associated with agronomic plants can be used to enhance the growth and yield of wheat.

Aarab *et al.* (2015) isolated 305 bacteria from the soil of rice in Northwestern Morocco. 136 isolates have phosphate solubilization ability, 17 best phosphate solubilizing rhizobacteria were selected on the basis of diameter of solubilization halos which are gram negative. Six isolates were found positive for IAA production while all isolates have ability to produce siderophore except P66. ACC deaminase activity was found in three isolates E34, E64 and E85 (*Pseudomonas* sp.). No isolates had ability for the solubilization of $FePO_4$ and $AlPO_4$ while seven isolates had ability for the solubilization $Ca_5HO_{13}P_3$and $CaHPO_4$. On the basis of sequencing of 16S rDNA, nine closely related to species belonging to three genera (*Aeromonas, Pseudomonas* and *Enterobacter*).

Park *et al.* (2005) screened nitrogen fixer bacteria from the soil of seven plants known as Sesame, Maize, Wheat Soybean, Lettuce, Pepper and Rice. They identified five isolates which showed nitroginase activity above 150 nmol/$h^{-1}mg^{-1}$ protien and on the basis of 16S rDNA sequencing, these isolates were identified as *Stenotrophomonas maltophilia* (PM-1 and PM-26), *Bacillus fusiformis* (PM-5 and PM-24), *Pseudomonas fluorescence* (PM-13). All isolates were positive for IAA production and in the presence of L-Tryptophan, IAA production was found minimum (100.4 µm/ml) by PM-13 (*Pseudomonas fluorescence*) while PM-24 (*Bacillus fusiformis*) produced maximum IAA (255 µmol/ml). *Bacillus fusiformis* showed highest nitroginase activity (3677.81 nmol/$h^{-1}mg^{-1}$protien). 99% idendity was observed between the 16S rDNA sequence of PM-1 and PM-26 with *Stenotrophomonas maltophilia*, PM-5 and PM-24 with *Bacillus fusiformis*, and PM-13 with *Pseudomonas fluorescence*.

Ahmad *et al.* (2008) isolated 72 bacteria from rhizospheric soil of crop plant. These isolates were biochemically identified. Phosphate solubilization was most frequently occurred in *Bacillus* (80 %) followed by *Azotobacter* (74.47 %), *Pseudomonas* (88.89%), and *Mesorhizobium* (16.67 %). IAA production was highest in the *Pseudomonas* followed by *Azotobacter* and *Bacillus* (7.03 µg/ml) at L-Tryptophan (500 µg/ml). *Azotobacter* (AZT-3, AZT-13, AZT-23), *Pseudomonas* (Ps-5) and *Bacillus* (B-1) showed highest antifungal activity against *Aspergillus, Fussarium* and *Rhizoctonia.*

Akhgar *et al.* (2014) observed that plants with biotic and abiotic stress produce ethylene from its immediate precursor 1- aminocyclopropane -1-carboxylase which reduces the root growth and cause senescence. Ethylene level is lowered down by ACC diaminase activity which breaks ACC to α-ketobutyrate and ammonia. During this study, 105 bacteria were isolated from rhizosphere of salt stressd canola. Out of 105 only 15 (*Pseudomonas fluorescence*) were found positive for ACC diaminase.

Saraf *et al.* (2013) analysed the biocontrol activity of PGPR against four phytopathogen of *Jatropha curcos* known as *Fusarium oxysporium, Macrophomina phasiolina, Aspergillus verscolor* and *Aspergillus nidulance*. Ten isolates of PGPR were isolated from agriculture field.

Seed of Jatropha after 5 day of incubation *P. putida* and *P. pseudoalcaligens* inhibited the growth of *M. phasiolina* by 90 and 45 %.

Iqbal (2013) isolated three Bacilli strains from different rhizospheric region of different crops plant because of their good survival rate under unfavourable condition. These isolates have potential of auxin production and promotion of plant growth in *Vigna radiate*. The effect of *Bacilli* in root morphogenesis was checked by *Arabidopsis thaliana*. Strain increased primary root length and lateral root density over control. *Vigna radiata* seeds with Bacilli isolates showed increase in root length, shoot length, fresh and dry weight.

Kumar *et al.* (2014) isolated 264 bacteria from soil. 12 isolates were found positive for phosphate solubilization from which MZPSB -207 showed highest solubilizing ability (864.71 µg/ml) and also showed IAA production (51.83 µg/ml), siderophore production, ammonia production, hydrolytic enzyme production ACC deaminase activity etc. Based on 16S rDNA sequencing all twelve isolates were identified which belong to four species of bacillus such as *B. substalis* (five isolates), *B. pumulis* (two isolates), *B. megaterium* (three isolates) and *B. amyloliquefaciens* (two isolates).

Bharucha *et al.* (2013) screened bacteria from rhizospheric soil of alfa alfa plant and on the basis of morphological and biochemical test they were identified as *Bacillus* species. The antagonist activity of Bacillus species were tested against *fusarium oxysporum* and *Aspergillus niger*. Siderophore were extracted through ethylene acetate yield 200 mg/ml.

Kamei *et al.* (2014) PTR-3 (*Pseudomonas aerugenosa*) was more effective biocontrol agent against *R. solani* which causing sheath blight disease of rice. PTR-3 isolate showed highest P-solubilizing activity (17.3 mg/50ml). PTR-1 was seen to have highest level of salicylic acid (0.54 mg/ml), siderophore (3.9 µmol benzoic acid/ml) and HCN. Highest phosphate solubility was observed in PTR-3.

Mohit (2013) isolated 10 IAA producing isolates, out of these 5 were used as good producers. Optimization for PGP traits were done at various cultural conditions of pH and temperature with various media component such as nitrogen source, tryptophan concentration. Isolate (mr) showed maximum IAA production in LB medium supplemented with tryptophan. The most suitable carbon source was glucose for *B. megaterium*, mannitol for *L. casei* and *B. substilis* glucose and sucrose for *L. acidophilous* while mannito and glucose for *B. cereus* for IAA production. Most suitable nitrogen source for IAA production was different with isolates type as $NaNO_3$ for *B. megaterium*, KNO_3 and peptone for *L. casei*, KNO_3 and peptone for *B. substilis* and *L. acidophilous* while $NaNO_3$ and peptone for *B.cereus*.

Rupaedah *et al.* (2014) screened one hundred forty four rhizobacteria from rhizosphere of sorghum rice and maize plants. Out of these only 25 rhizobacteria were seen to increase the growth and chlorophyll content of sorghum. The highest concentration of IAA was seen by LR73 isolate while ML14 and LR73 produced higher amount of GA. The higher concentration of cytokinin was produced by LR73. By molecular characterization, LR73 was identified as *Mycobacterium senegalense*.

Mia *et al*. (2012) found that the seed germination and seedling vigour, root growth, seedling emergence, root growth significantly increased when seeds of rice were inoculated with PGPR strains UPMB10 (*Bacillus sphaericus*), *rhizobium* strains SB16, UPMR1006, UPMR1102. Out of these UPMB10 showed better seedling growth and strain UPMR1006 profuse hair in redical.

Aiechour *et al*. (2012) evaluated fourteen bacterial isolates which were characterized as *Fluorescent chlororaphis* from the rhizospheric soil of potato plants. These strains inhibit the growth of phytopathogenic fungi (*Fusarium oxysporum. F. lycopersici*, *F. oxysporum, F. albedinis, F. solani*, *Rhizoctonia solani* and *Pythium ultimum*). The extract obtained from *F. chlororaphis* inhibits the growth of all these fungi into dual culture.

Sedeghi *et al.* (2012) observed that when the concentration of NaCl was increased up to 300 mM, dry wight and cfu/ml of *Strepyomyces* isolates increased significantly. IAA production of this isolates wad 2.4 µg/ml. The amount of IAA was reached up to 4.7 µg/ml by adding NaCl 300 mM concentration. Phosphate solubilization was decreased in the presence of NaCl while siderophore production was increased. The treatment of this isolate showed significant incensement in seed germination, shoot length, and dry weight of wheat.

Upadhyay *et al.,* (2009) isolated 130 rhizobacteria from wheat rhizosphere which grown in saline soil and tested for PGR at higher salt concentration (2, 4, 6 and 8%). Only 24 isolates could tolerate at 8 % NaCl and produced IAA while 10 solubilized to phosphate, 8 produced siderophore and six able produced GA. Only three isolates were able to produced ACC deaminase. Tolerable isolates were identified as *Bacillus* sp. by 16S rDNA sequencing.

Forty four strain of *Bacillus* were isolated from the rhizosphere of tomato for some fungi that cause plant disease (*Botritis cineria, Pythiumultimum, fusarium oxysporium, Cladosporium cucumerinum, Aspergillus niger*). The isolates were screened for hydrolytic enzyme activities such as protease, chitinase and lipase. These isolates were found to have antagonistic activities against pathogenic fungi, where four among them were used as strong inhibitor. (Venant *et al.,* 2013).

Gamit and Tank, (2014) isolated bacteria from rhizospheric soil of *Cajanuscajan* and identified by 16S rDNA sequencing which showed maximum relativity with *Pseudomonas pseudoalcaligenes*. These isolates were tested in pot experiment with *Cajanun cajan*, showed significance increasement in root length, shoot length and biomass.

Proposed Methodology

1 Isolation and characterization of plant growth promoting rhizobacteria

(A) Site description and soil collection

Soil sample will be collected from rhizospheric region of crop plant from different agricultural location of Dayalbagh, Agra. Soil sample will be collected from depth of up to 20 cm after removing approximately 3 cm of the soil surface, then Soil sample will be labeled properly and stored at 4°C till futher use (Lamsal *et al.,* 2012).

(B) Isolation of rhizobacteria

For isolation of rhizobacteria, 10 g soil will be suspended in 90 ml sterile water and agitated for 30 minutes on rotatory shaker. 1 ml of this stock will be diluted up to 10^{-8} and then 0.1 ml of diluted sample will be spread on specific medium.

(C) Morphological and Biochemical characterization

For preliminary identification rhizobacterial isolates will be examined according to methods describe in *Bergey's Manual of Systematic Bacteriology* (Holt *et al.,* 1994).

(D) Characterization of potential rhizobacterial isolates at molecular level

(i) Broth cultivation of bacterial isolates: The genomic DNA from each of the bacterial isolates will be extracted by standard procedure.

(ii) Amplification of 16S rDNA: Amplification of 16S rDNA will be done using universal primer 27 F and 1492 R or other suitable primers.

(iii) Sequencing of 16S rDNA: The amplified 16S rDNA will be purified using PCR purification kit and purified product will be out sourced for sequencing.

(iv) Bioinformatics analysis: Sequencing will be compared with a database that is available in NCBI using BLAST search engines (http://www.ncbi.nlm.nih.gov) to obtain accession number. A phylogenetic tree will be created using the program MEGA- 4 by comparing

the DNA sequences of multiple bacterial species obtained from the gene in the NCBI database.

2 Standardization of media composition for multiplication of plant growth promoting rhizobacteria. The following parameters will be optimized.

(a) pH

(b) Temperature

(c) Nitrogen source

(d) Carbon source

(e) NaCl concentration

The bacterial growth will be quantified by estimating maximum specific growth rate and growth yield coefficient.

3 *In vitro* screening of rhizobacterial isolates for their plant growth promoting activities

(a) Quantitative assay for phosphate solubilization: Determination of phosphate solubilization will be done by method of Nautiyal, (1999) using U V spectrophotometer.

(b) Production of Indol-3-acetic acid: Determination of Production of IAA will be done by method of Loper and Scroth, (1986) using U V spectrophotometer.

(c) Production of siderophore: Determination of Production of siderophore will be done by method of Leong *et al.* (1986) using U V spectrophotometer.

4 Effect of potential plant growth promoting rhizobacteria in certain field grown crop plants

(a) Analysis of various physico-chemical characteristics of soil.

(b) Evaluation of the effect of inoculant on plant root length, shoot length, and plant biomass and crop yield.

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